

Identification, Characterization, and Physiological Actions of Factor H as an Adrenomedullin Binding Protein Present in Human Plasma

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ABSTRACT A recently discovered adrenomedullin binding protein has been characterized as complement factor H, an important regulator of the complement cascade. This review will describe the evidence that led to the identification of factor H as an adrenomedullin binding protein and will address the implications that such binding has in the radioimmunoassay of AM in plasma. We will also describe the possible physiological implications of AM binding: namely, factor H suppresses the antimicrobial activity of AM, enhances AM-mediated induction of cyclic-AMP in rat fibroblasts, and augments the AM-mediated growth of a human cancer cell line. These initial studies suggest that factor H may be an important factor in the regulation of AM physiology. The elucidation of the mechanisms that modulate AM activity will be necessary for the understanding of the role of AM in normal and pathological conditions. *Microsc. Res. Tech.* 57:23–27, 2002. © 2002 Wiley-Liss, Inc.

INTRODUCTION

Circulating proteins that specifically bind to a peptide can provide a control for its biological activity. These binding proteins may affect peptide distribution (i.e., transport to the interstitial space) and availability in proximity to its receptor, and may also modify its metabolic clearance. The existence of binding proteins is a relatively common event. Several peptide growth factors are bound specifically to binding proteins. For example, the insulin-like growth factors (IGF-I and IGF-II), peptides that regulate the proliferation and differentiation of several cell types, are present in the circulation and throughout the extracellular space bound to members of the IGF binding protein (IGFBP) family. The half-lives of the IGFs are dependent on their association with the IGFBPs. The IGFBPs also act as transport proteins in plasma, regulate the tissue distribution of IGFs, and can either inhibit or augment the metabolic and mitogenic effects of IGFs by modulating their interaction with their receptors (Clemmons, 1998).

In this paper, we will describe the identification of a novel binding protein for adrenomedullin (AM). AM is a peptide present in a variety of mammalian species and involved in the regulation of several physiological functions, including: cardiovascular tone, central brain activity, bronchodilation, renal function, hormone secretion, cell growth, differentiation, and immune response (Hinson et al., 2000). We will also discuss the implications that protein binding of AM has in procedures for AM quantitation and in the modulation of some AM activities.

DISCOVERY AND CHARACTERIZATION OF THE AMBP-1

The development of methods for AM quantitation in plasma has encountered interferences similar to those

produced by the IGFBPs on the quantitation of IGF (Underwood et al., 1982). The standard method for measurement of AM in human and animal plasma requires an extraction step with Sep-Pak C-18 cartridges prior to the quantitation of AM by radioimmunoassay (Kitamura et al., 1994). Without this step, the displacement of ¹²⁵I-AM from the primary antibody by increasing concentrations of plasma is not parallel to the displacement slope of synthetic AM in the assay standard curve. Also, the recovery of ¹²⁵I-AM is usually higher than the total binding (B₀) in the assay (Elsasser et al., 1999a). In a Sephadex G-50 chromatography, the elution pattern of ¹²⁵I-AM added to plasma shifts from a single peak of approximately 6 kDa to a dual peak with a high molecular entity (>40 kDa) eluting at the column void volume (Elsasser et al., 1999a). Finally, while the AM from plasma is not altered by up to four freeze-thaw cycles, recovery of exogenous AM is markedly reduced after a single freeze-thaw cycle, suggesting that a protein may be present in circulation that can bind AM with low affinity, aiding in its stability (Lewis et al., 1998).

The existence of an AM binding protein was demonstrated for the first time using a radioligand blotting procedure similar to that described for the IGFBPs. In this procedure, plasma proteins are fractionated by SDS-PAGE under non-reducing conditions, transferred to nitrocellulose, and incubated in the presence of ¹²⁵I-labeled peptide (Hossenlopp et al., 1986). This technique, applied to the detection of AM binding proteins in the plasma of several species, revealed the existence of protein(s) capable of binding AM in a specific and

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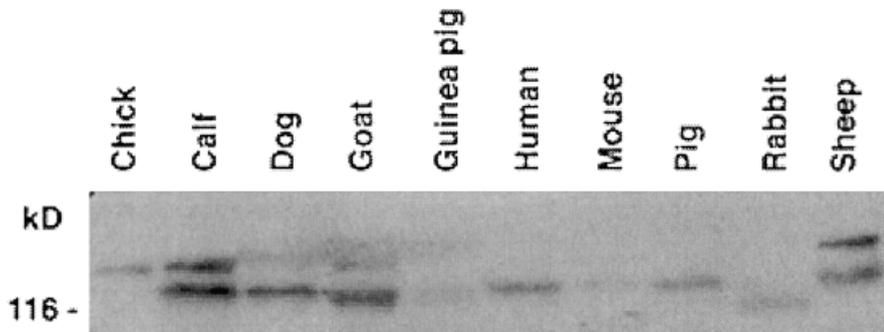


Fig. 1. Detection of AM binding proteins by radioligand blotting in the plasma of different species. The proteins have an apparent molecular weight of 120 and 140 kDa. Reproduced from Elsasser et al. (1999) with permission of the publisher.

competitive fashion (Elsasser et al., 1999a). All species analyzed (chick, calf, dog, goat, guinea pig, human, mouse, pig, rabbit, and sheep) contained a 120-kDa binding protein denoted AMBP-1. The plasma from ruminant species (calf, goat, and sheep) contained an additional band with an apparent molecular weight of 140 kDa (Fig. 1).

Although important for the initial detection of AMBPs in plasma, some problems were associated with the radioligand blotting method. The reproducibility of the technique was not entirely satisfactory and important differences were found between different batches of the human ^{125}I -AM. A non-radioactive ligand blotting assay using AM labeled with fluorescein has been demonstrated to give a better reproducibility and sharper band formation than the use of ^{125}I -AM (Pío et al., 2001). The explanation for this fact may be found in the mild conditions used for the non-radioactive labeling procedure. It is also possible that labeling in certain positions may disrupt the structure of the peptide and affect the efficiency of binding. Figure 2 shows the potential amino acids labeled with each method. It is evident that the radioactive procedure can label the amidated tyrosine at the C-terminal of AM, a position that is critical for AM activity (Eguchi et al., 1994) and may be also important for its interaction with the AM binding protein.

Using the non-radioactive ligand blotting, together with HPLC/SDS-PAGE purification techniques, we isolated AMBP-1 and characterized it by amino acid composition analysis, amino acid sequencing, MALDI/MS and MS/MS. A database comparison of the results identified AMBP-1 as human complement factor H (Pío et al., 2001).

COMPLEMENT FACTOR H: STRUCTURE AND FUNCTIONS

Factor H is an important regulator in the activation of the complement system. This system consists of an array of proteins (proteolytic enzymes, regulatory proteins, and proteins capable of causing cell lysis) that play a key role in the elimination of pathogens and the initiation of inflammatory responses (Liszewski and Atkinson, 1993). Complement is the major non-cellular system of innate immunity in human and can be activated via three distinct pathways: the classical, the alternative, and the lectin pathway (Zhang et al., 1999). The activation of the complement cascade is highly controlled; several regulatory proteins exist that function to prevent uncontrolled complement activa-

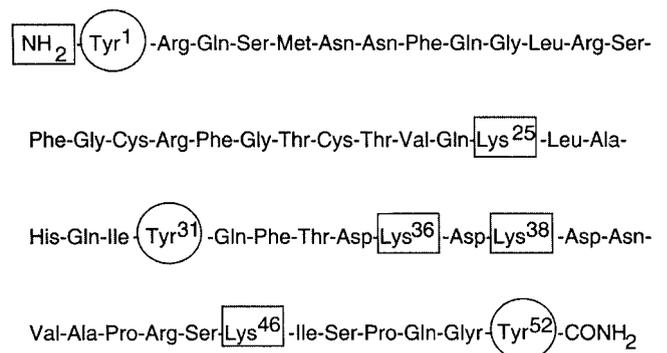


Fig. 2. Amino acid sequence of AM showing the positions of lysine and tyrosine residues, sites susceptible to labeling by succinimidyl esters or radioiodination, respectively. Lysine residues and the N-terminal group are encircled and tyrosine residues are boxed.

tion. In this sense, the alternative pathway is spontaneously activated and needs to have specific protection mechanisms to restrict the destructive effects of the activated system (Müller-Eberhard and Schreiber, 1980). Factor H is a key regulatory protein in the activation of this pathway. Factor H binds to C3b, the key component for complement activation, displacing Bb from the C3 convertase, and therefore destroying its activity. Factor H is also a necessary cofactor for the inactivation of C3b by factor I. Therefore, the final result of the factor H activities is the inhibition of the alternative pathway of complement (Harrison and Lachmann, 1980; Sim et al., 1981; Whaley and Ruddy, 1976).

Factor H is a 150-kDa glycoprotein present in human plasma in a concentration of about 500 $\mu\text{g}/\text{ml}$ (Whaley, 1976). It is constitutively produced by the liver, and is also synthesized extrahepatically by mononuclear phagocytes, fibroblasts, endothelial cells, mesangial cells, astrocytes, oligodendrocytes, and neurons (Friese et al., 2000; Schwaeble et al., 1987). The complete sequence of human factor H is already known (Ripoche et al., 1988). Factor H is composed of 20 repetitive domains termed short consensus repeats (SCR), each approximately 60 amino acids in length. It behaves as an extended molecule in solution and appears to have a structure resembling the beads on a string (DiScipio, 1992). Factor H belongs to a protein family whose members are defined by common structural and functional characteristics. Other members include factor

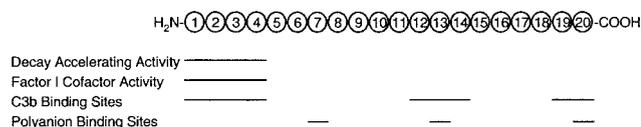


Fig. 3. Structure of factor H and location of its functional domains. The protein is composed of 20 short consensus repeats (SCRs) resembling the beads on a string. Each SCR has four conserved cysteine residues that form two disulfide bonds per SCR. The location of the known functional domains are indicated.

H-like protein (FHL-1), factor H related protein 1 (FHR-1), FHR-2, FHR-3, and FHR-4 (reviewed by Zipfel et al., 1999). FHL-1 is a 42-kDa protein processed from the factor H gene by alternative splicing. The protein is identical in sequence to the seven N-terminal domains of factor H and possesses four additional unique amino acids at the C-terminal end. FHL-1 shares the complement regulatory functions of factor H. The FHRs are composed of 4 and 5 SCRs and their functions remain unclear.

Structure-function analysis of factor H demonstrates that the domains required for the complement regulatory activity are located within the four N-terminal SCRs (Fig. 3). Three C3b binding domains have been identified on factor H (Jokiranta et al., 1996; Sharma and Pangburn, 1996). Each of the three binding sites interacts with a distinct site on C3b (Jokiranta et al., 2000). Three binding sites for polyanions, such as heparin and sialic acid, have been identified within the protein. Two of these sites have been localized to SCRs 7 and 20 (Blackmore et al., 1996, 1998), and the other is located in or near SCR 13 (Pangburn et al., 1991). The binding of factor H to this polyanions at the cell surface may provide a mechanism for discrimination between complement activator and non-activator surfaces.

In addition to its regulation of complement activation, other functions have been identified for factor H. It is a ligand for L-selectin (Malhotra et al., 1999) and also binds to the integrin Mac-1 (C11b/CD18), enhancing the activation response of human neutrophils (DiScipio et al., 1998). Factor H also induces the secretion of interleukin 1 β by monocytes (Iferroudjane et al., 1991), and acts as a chemotactic protein for them (Nabil et al., 1997). Finally, factor H binds to cell surface components of several pathogens (Díaz et al., 1997; Horstmann et al., 1988; Neeleman et al., 1999; Ram et al., 1998a,b), inhibiting the alternative pathway of complement and thus enhancing their pathogenicity.

IMPLICATIONS OF FACTOR H IN THE QUANTITATION OF AM IN PLASMA

The determination of AM in plasma has been a challenge, largely because the peptide circulates at relatively low concentrations in plasma and, like amylin, has a tendency to adhere non-specifically to surfaces (Cooper, 1994; Lewis et al., 1998). Several groups have developed immunoassays for the measurement of AM in plasma. In general, there is a certain consistency between the different methodologies in terms of the concentration of circulating AM (Hinson et al., 2000). The normal range for plasma AM concentrations in

healthy human donors has been established as 1 to 10 pM (Hinson et al., 2000). Possible factors affecting the performance of AM assays, and the pitfalls associated with measurements of the peptide, have been analyzed in detail (Lewis et al., 1998). One of the main problems of AM assays is inconsistency in the extraction method prior to radioimmunoassay. An assay buffer containing Triton X-100 and alkali-treated casein prevents the adsorption of AM to surfaces, thereby reducing non-specific binding and improving the recovery of the peptide and the precision of the assay. For peptide extraction, plasma is loaded onto a Sep-Pak C-18 cartridge and AM is eluted with isopropanol/HCl (Lewis et al., 1998). Although factor H is more hydrophobic than AM and, therefore, should be better retained by the reverse-phase matrix, we have found that this process eliminates factor H from the extract to be analyzed (Elsasser et al., 1999a; Pío et al., 2001). The factor H molecule has a contour length of 495 Å and a cross-sectional diameter of 34 Å and folds on itself, thus reducing the length of the protein and increasing its width (DiScipio, 1992). The elongated structure of factor H accounts for its large apparent size as determined by gel filtration (Pangburn, 2000), and suggests that factor H is too big to penetrate the particle pores in the Sep-Pak cartridge (125 Å). If factor H passes through the cartridge, any AM bound to factor H will not be retained in the column and the efficacy of the extraction protocol will be reduced. If factor H carries a certain fraction of the total AM pool, the protocols for AM quantitation will only measure the free AM circulating in plasma. We have confirmed this fact by demonstrating the presence of a significant amount of AM in the unbound fraction after the extraction (Pío et al., 2001). AM concentrations are known to change in several diseases states (Hinson et al., 2000). The involvement of AM in the response to severe disease stress has been implied through measured increases in circulating plasma concentrations of AM during sepsis and after endotoxin challenge (Elsasser et al., 1999b; Hirata et al., 1996; Shoji et al., 1995). At the same time, calves undergoing an acute phase response to a parasitic infection (*Sarcocystis cruzi*) present a reduced expression of factor H (detected as AMBP-1) in plasma when compared to uninfected controls (Elsasser et al., 1999a). This suggests that changes in circulating free AM may also be dependent on changes in its binding protein concentration. For these reasons, the determination of both factor H and total AM in plasma will be important in understanding the role of AM in physiological and pathological conditions. At the moment, the low levels of AM detectable in plasma had led to the conviction that AM is not a functional hormone, although it may act as an autocrine/paracrine factor in the neighborhood of its secreting sites. If total AM levels in plasma are prone to be significantly higher than those currently measured, that would reinforce the possibility of a physiological role for circulating AM.

PHYSIOLOGICAL IMPLICATIONS OF AM BINDING

As previously indicated, the presence of a binding protein can modify the concentration and availability of a factor in biological fluids. This protein can alter the

half-life of the factor, and may either stimulate or inhibit its activity. The binding of AM to factor H can block the non-receptor mediated AM antimicrobial activity on *Escherichia coli*. It can also induce two receptor-mediated activities: increased cAMP production in Rat-2 fibroblasts, and growth of the T-47D human breast cancer cell line (Pío et al., 2001). At present, only speculation can be made about the mechanisms governing these processes. If AM exerts its antimicrobial effect by forming pores on the plasma membrane (Allaker et al., 1999), factor H could act as a sequestering molecule to reduce the binding of AM to the pathogen membrane. The induction of AM activity by factor H in the two receptor-mediated events is more difficult to explain. Factor H contains three binding sites for polyanions that mediate its binding to sialic acid-containing cell surfaces. On the other hand, factor H interacts with specific receptors on a variety of cell types (Avery and Gordon 1993; DiScipio et al., 1998). We have suggested (Pío et al., 2001) that factor H may bind to the cell surface, thus acting as a carrier of AM and bringing it into closer proximity to its membrane receptor. That would explain why the AM-mediated induction of cAMP in Rat-2 cells is modified by factor H without affecting the kinetics of binding of AM to its receptor (Pío et al., 2001). It is also possible that factor H increases AM stability by preventing its degradation by proteases present in the cell membrane (Lewis et al., 1997).

Some evidence has suggested that AM could have immunomodulatory actions. In the immune system, AM is produced by pulmonary and peripheral blood macrophages and may modulate their function (Martínez et al., 1995; Kubo et al., 1998a,b). In LPS-stimulated rat alveolar macrophages, AM inhibits the secretion of cytokine-induced neutrophil chemoattractant (Kamoi et al., 1995). AM is also produced and secreted by RAW 264.7, a murine monocyte/macrophage cell line. In RAW 264.7, AM increases the secretion of tumor necrosis factor- α (TNF- α), and suppresses the LPS-mediated secretion of TNF- α and interleukin-6 (Kubo et al., 1998b). On the other hand, AM is expressed by mucosal surfaces and has antimicrobial properties (Allaker et al., 1999; Walsh et al., 1996). AM may also interfere with the regulatory action of factor H in complement activation. As shown in Figure 4, the cofactor activity of factor H in the degradation of C3b by factor I is augmented by the presence of AM (Pío et al., 2001). This novel mode of regulation in complement activation could provide a new link between the hormonal and immune systems.

CONCLUSIONS

AM is involved in multiple physiological and pathological processes (Hinson et al., 2000). The elucidation of the mechanisms that regulate its availability in tissues, and the factors that modulate its activity, are crucial for the understanding of its biological effects. Factor H, as an AM binding protein, has been implicated in the regulation of AM function and has been shown to interfere with the quantitation of AM in plasma. We have also suggested that the expression pattern of factor H may alter the availability of AM in states of health and disease. Therefore, the quantitation of factor H and the total AM concentration in

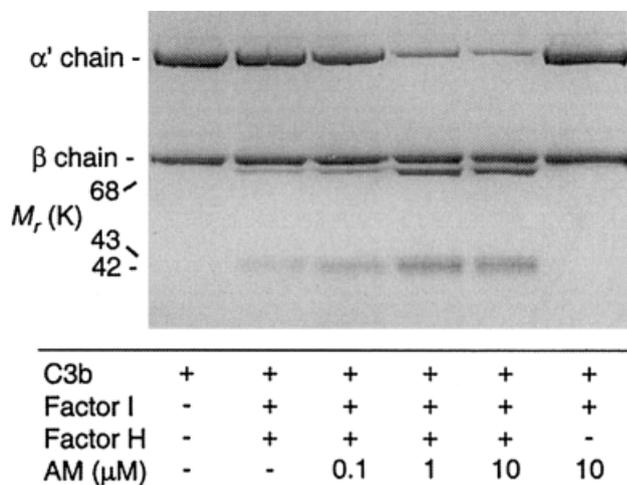


Fig. 4. Effect of AM in the cofactor activity of factor H. C3b is composed of a 104-kDa α' chain and a 71-kDa β chain. The cleavage of the C3b α' chain by factor H and factor I produces three bands with M_r 68K, 43K, and 42K. Addition of AM to the reaction causes an increase in the cleavage of C3b. As the levels of AM are increased in the reaction mixture, there is a parallel increase in the split product formation, with a reciprocal reduction in the 104-kDa band. AM has no activity in the absence of factor H.

plasma (vs. the free AM currently measured) may be important for the elucidation of the role of AM in several disease states. The major circulating form of AM is the inactive C-terminal glycine-extended form, which can be converted to mature AM by enzymatic amidation (Kitamura et al., 1998). It will be interesting to determine if factor H specifically binds to mature AM, or also binds to glycine-extended AM. In addition, the binding of AM to other members of the factor H family (FHL-1 and FHRs) needs to be determined. On the other hand, the regulation of factor H activity by AM suggests that the peptide may be involved in the control of complement activation, and may open a novel research avenue in AM physiology.

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